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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Gary Ruvkun et al.   Art Unit: 1632
Serial No.: 08/908,453   Examiner: R. Shukla
Filed: August 7, 1997   Customer No.:   21559
Title: AGE-1 POLYPEPTIDES AND RELATED MOLECULES AND
             METHODS

Assistant Commissioner For Patents
Washington, DC 20231

DECLARATION OF DR. GARY RUVKUN UNDER 37 C.F.R. § 1.132
TRAVERSING GROUNDS OF REJECTION

Under 37 C.F.R. §1.132, I declare:

1. I am an inventor of the subject matter described and claimed in the above-captioned patent application.

2. I have read the Office Action mailed on June 7, 2001.

3. The conclusion that *age-1* is a PI 3-kinase is supported by at least the following evidence: (i) sequence homology and characteristic structural motifs, (ii) physical interactions, (iii) genetic interactions, and (iv) biochemical evidence.

4. *age-1* shares sequence homology with other PI 3-kinases. *C. elegans* AGE-1 is the worm protein most homologous to known PI 3- kinases from mammals, and AGE-1

displays structural motifs characteristic of PI 3-kinases, including a p85 interaction

domain and a lipid kinase domain. The closest mammalian homolog of *C. elegans* AGE-

1 is mammalian p110 PI 3-kinase. When the p110 sequence was used to search the worm

proteome database, AGE-1 was found to be p110's closest homolog. The random

probability of alignment of AGE-1 with mammalian p110 kinase was extremely low, less

than e^{-100}. Moreover, when the same search was conducted with a mammalian p110 PI 3-

kinase query (for example, XP_066258, phosphoinositide 3-hydroxykinase p110-alpha

subunit *Homo sapiens*), the next closest sequence hit in the *C. elegans* proteome database

was <u>30-logs</u> lower in probability, an enormous step down in sequence alignment terms.

In addition, when the *C. elegans* AGE-1 sequence was used to search a mammalian

proteome database, mammalian PI 3-kinases were also found to be AGE-1's closest

homologs. Again, the random probability of this alignment to occur by chance rather

than to reflect true orthology was extremely low, less than e^{-98}. These results provide

strong evidence that *age-1* is the *C. elegans* ortholog of biochemically characterized

mammalian PI 3-kinases.

Also consistent with these high levels of sequence similarity, we have found that

substitution of an amino acid conserved between the AGE-1 polypeptide and mammalian

p110 PI 3-kinase leads to complete loss of AGE-1 activity. This result lends further

credence to the biological relevance of the sequence shared between AGE-1 and

mammalian p110.

5. As a PI 3-kinase, AGE-1 would be expected to exhibit the characteristic

physical interactions of a PI 3-kinase. PI 3-kinases are heterodimeric enzymes that

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consist of a catalytic subunit, p110, and an SH2-domain-containing adapter subunit. In vertebrates p110 interacts with p85 or p55, SH2-domain-containing adapter subunits, through its amino-terminal domain; this interaction is required to activate the catalytic activity of p110. Examining the AGE-1 sequence, the amino-terminal domain of AGE-1 and p110 are 25% identical, suggesting that this interaction domain is under selective pressure to remain the same, perhaps due to the presence of a common regulatory partner, such as p85 or p55.

We used a BLAST search of the *C. elegans* genome to identify p85 or p55 homologs. This search identified a single *C. elegans* polynucleotide, *y110a7a-2.k*, that shared significant sequence homology (random probability of alignment: 3.8×10^{-29}) with mammalian p85 and p55. *y110a7a-2.k* encodes a p55-like adapter subunit, and was subsequently renamed *aap-1* for *age-1* adapter protein-1. Sequence comparisons using BLAST and PILEUP algorithms (Genetics Computer Group, WI) showed that the structure of AAP-1 was most closely related to SH2 domains from other Class IA PI 3-kinase adapter subunits. Based on the mammalian PI 3-kinase interactions, we predicted that the AAP-1 PI 3-kinase adapter subunit would bind to the amino-terminal domain of AGE-1.

We tested this prediction by producing recombinant AAP-1 and assaying for AAP-1 binding to the amino-terminal domain of AGE-1. AGE-1 (amino acids 1-268) was efficiently co-precipitated by AAP-1-containing beads; AGE-1 failed to bind to beads lacking AAP-1. These results indicated that AGE-1 specifically interacts with AAP-1, and confirmed our prediction that the interaction between mammalian p110 and

p85 is conserved in their *C. elegans* counterparts, AGE-1 and AAP-1. AGE-1's

interaction with AAP-1 also provides support for AGE-1's identification as a PI 3-kinase,

because of its shared physical interaction with SH2 adapter proteins.

This physical interaction was further demonstrated by the following *in vivo*

experiment. If *aap-1* encodes the authentic regulatory subunit for AGE-1 PI 3-

kinase, then *aap-1* gene function should be required for *age-1* function *in vivo*. To

test this prediction, RNA-mediated interference was used to reduce *aap-1* gene

function. Specifically, in *C. elegans,* injection of double-stranded RNA of a target

gene results in RNA-mediated interference (RNAi) with target gene expression.

This interference effectively reduces or eliminates the gene's activity in the

injected animal and its progeny.

In our experiment, RNAi was used to test the prediction that animals with

decreased *aap-1* function would resemble *age-1* loss-of-function mutants, *i.e.,*

display constitutive arrest at the dauer larval stage. These studies were carried out

in a sensitized genetic background that allowed the detection of small decrements

in *aap-1* activity. We found that, in a sensitized background, *aap-1* RNAi strongly

enhanced dauer arrest (compared to that observed for uninjected control animals),

as expected if *aap-1* gene function is required for *age-1* function *in vivo*.

This physical and genetic evidence indicates that the SH2-domain-

containing adapter protein, AAP-1, interacts with AGE-1, consistent with the

interaction of their mammalian homologs and consistent with the identification of

AGE-1 as a PI 3-kinase.

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6. Additional evidence that AGE-1 is a PI 3-kinase is provided by genetic interactions that place AGE-1 in the insulin pathway. Vertebrate PI 3-kinases function in insulin signaling. If AGE-1 functions as a PI 3-kinase, then by analogy to mammalian systems AGE-1 would be predicted to act downstream of the *C. elegans* insulin receptor tyrosine kinase, which has specific phosphotyrosine motifs (YXXM) associated with p85 SH2-domain binding. We tested this prediction genetically and found that, not only does AGE-1 function downstream of the *C. elegans* insulin receptor, but AGE-1 also functions upstream of PDK and AKT kinases. This is relevant because PDK and AKT kinases have pleckstrin homology domains that are specifically regulated by the product of AGE-1, that is, PIP3.

Moreover, our genetic studies with *daf-18*, another component of the insulin-like signaling pathway, provide further support for AGE-1's identification as a PI 3-kinase. *daf-18*, the *C. elegans* homolog of mammalian PTEN, encodes a lipid phosphatase that dephosphorylates phosphoinositides *in vitro* and lowers PIP3 levels *in vivo* by inhibiting PIP3 accumulation in response to insulin signaling. Since PTEN dephosphorylates PIP3, DAF-18 may normally function to decrease the PIP3 output of AGE-1 PI 3-kinase signaling. If so, mutations in *daf-18* should suppress mutations in *age-1*. In our experiments, this hypothesis was found to be correct; we determined that mutations in *daf-18* suppressed mutations in *age-1*. Moreover, we found that loss of DAF-18 enhanced PIP3 signaling to
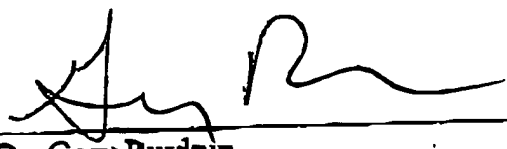
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AKT kinases, consistent with AGE-1 generating a PIP3 second messenger and again consistent with AGE-1's role as a PI 3-kinase.

7. Finally, as biochemical evidence that AGE-1 is a PI 3-kinase, we again direct the Examiner's attention to the previously submitted publication by Babar et al. (*Neurobiology of Aging* 20:513, 1999). In this reference, the authors treated *C. elegans* with a known chemical inhibitor of mammalian PI 3-kinases, a chemical termed LY294002. This treatment mimicked the effects of AGE-1 mutations (pages 516-517), as measured by dauer formation, thermotolerance, and life span. This experiment indicates that the *in vivo* outcome of a loss of AGE-1 function parallels the *in vivo* outcome of a loss of PI 3-kinase activity. This biochemical result is therefore consistent with AGE-1 functioning as a PI 3-kinase.

8. It is reasonable to believe that PI 3-kinase activity may be readily assayed in *C. elegans* extracts. To prepare a *C. elegans* homogenate for a PI 3-kinase assay, standard methods of extract preparation are employed. In particular, worms are washed and concentrated, followed by homogenization to break open their outer cuticles. The homogenate is then centrifuged to remove debris, and the extract is used in a standard PI 3-kinase assay, such as the assay referenced in the specification at page35, lines 25 and 26. Alternatively, if desired, AGE-1 protein may be purified from the crude homogenate using routine methods of protein purification. All of these methods were standard in the art at the time the patent application was filed.

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9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 3/8/02

Dr. Gary Ruvkun